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Forced evolution of glutathione *S*-transferase to create a more efficient drug detoxication enzyme

(drug resistance/mechlorethamine/hematopoietic stem cells/alkylating agents/gene therapy)

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ABSTRACT **Glutathione *S*-transferases (EC 2.5.1.18) in mammalian cells catalyze the conjugation, and thus, the detoxication of a structurally diverse group of electrophilic environmental carcinogens and alkylating drugs, including the antineoplastic nitrogen mustards. We proposed that structural alteration of the nonspecific electrophile-binding site would produce mutant enzymes with increased efficiency for detoxication of a single drug and that these mutants could serve as useful somatic transgenes to protect healthy human cells against single alkylating agents used in cancer chemotherapy protocols. Random mutagenesis of three regions (residues 9–14, 102–112, and 210–220), which together compose the glutathione *S*-transferase electrophile-binding site, followed by selection of *Escherichia coli* expressing the enzyme library with the nitrogen mustard mechlorethamine (20–500 μM), yielded mutant enzymes that showed significant improvement in catalytic efficiency for mechlorethamine conjugation (up to 15-fold increase in k_{cat} and up to 6-fold increase in k_{cat}/K_m) and that confer up to 31-fold resistance, which is 9-fold greater drug resistance than that conferred by the wild-type enzyme. The results suggest a general strategy for modification of drug- and carcinogen-metabolizing enzymes to achieve desired resistance in both prokaryotic and eukaryotic plant and animal cells.**

The glutathione *S*-transferases (EC 2.5.1.18) are a family of enzymes that are responsible for the detoxication of a broad class of electrophiles. In both prokaryotic and eukaryotic cells, these enzymes have been shown to catalyze the conjugation of the tripeptide glutathione to a variety of compounds, resulting in products that are generally less reactive (1–3). High levels of expression of several related glutathione *S*-transferase isozymes enable these enzymes to protect the cell from numerous structurally diverse electrophiles present in our environment (4–6) and used in cancer chemotherapy (1, 2).

Several reports have shown that chronic treatment of cultured cells (7, 8) or clinical tumors (9) with alkylating agents results in resistant subpopulations of cells that express alpha class glutathione *S*-transferases at levels higher than those in the sensitive parental populations. In studies where transfected cDNAs were used to compare cell populations that differed in the single variable of glutathione *S*-transferase expression, the recombinant rat 1-1 isozyme conferred as much as 3-fold resistance to chlorambucil in mouse fibroblasts (10), and this fold resistance overlaps the fold resistance seen in clinical studies of nitrogen mustard resistance (11, 12). Consistent with the observed drug resistance, glutathione *S*-transferases have been shown to catalyze the conjugation of the nitrogen mustards chlorambucil and melphalan to glutathione (13–15).

Each subunit of a glutathione *S*-transferase dimer contains an independent active site composed of a G site for binding glutathione and an H site for binding hydrophobic electrophiles (2). Each subunit contains two domains with residues primarily from domain I forming the G site (16–18), a result confirmed by mutagenesis studies (19, 20). Protein regions that form the H site were identified by the cocrystallization of the enzyme with reaction products. The H site (Fig. 1), a cavity located between the two domains of each subunit, is formed by (*i*) residues that are located in the first 10 amino acids of the protein and which lie adjacent to the catalytically important tyrosine-6 residue (16), (*ii*) residues from one face of α-helix D, and (*iii*) residues from the extreme carboxyl-terminal tail of the protein (18, 21–23). An amino acid alignment (Fig. 2) illustrates the relative locations of these three regions of amino acids which form the H site (18, 21–23).

The amino acids which compose the H site in wild-type glutathione *S*-transferases have generally enabled these enzymes to bind and conjugate a variety of structurally diverse substrates with moderate efficiency. We proposed (2, 24) that random mutagenesis of the H-site residues, when combined with selective pressure for detoxication of a single electrophile, would identify glutathione *S*-transferase variants that were more efficient catalysts and which would be better able to protect a cell against a single electrophile. Recombinant glutathione *S*-transferases with such properties might find use as somatic transgenes capable of protecting normal human cells against the single alkylating agents often used in cancer chemotherapy regimens.

In this paper, we describe a strategy which yields a specific, efficient, mutant, drug-metabolizing enzyme which was selected from a library of randomly mutated derivatives of a broad-specificity drug-metabolizing enzyme, glutathione *S*-transferase. We demonstrate that the increased catalytic efficiency is a result of a higher turnover rate for the conjugation reaction with little change in the K_m for the hydrophobic electrophilic substrate mechlorethamine.

METHODS

Plasmid Construction. We inserted the glutathione *S*-transferase 2 cDNA into the pUC120 plasmid (25). The promoter-coding region cassette from this plasmid was amplified by using the polymerase chain reaction and inserted into the multiple cloning site of a modified pAlter plasmid (Promega). Because the pAlter plasmid contains a P_{lac} promoter identical to the one in our promoter-cDNA cassette, we deleted the 213-bp *Hin*dIII–*Pvu* II fragment containing the P_{lac} promoter; the modified plasmid was designated pAMG88. The 843-bp promoter-cDNA cassette was inserted into the *Eco*RI and *Bam*HI sites of the pAMG88 multiple cloning site to generate pAMG207. Ampicillin-resistant versions of these plasmids were designated pAMG88a and pAMG207a.

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Abbreviation: CDNB, 1-chloro-2,4-dinitrobenzene.

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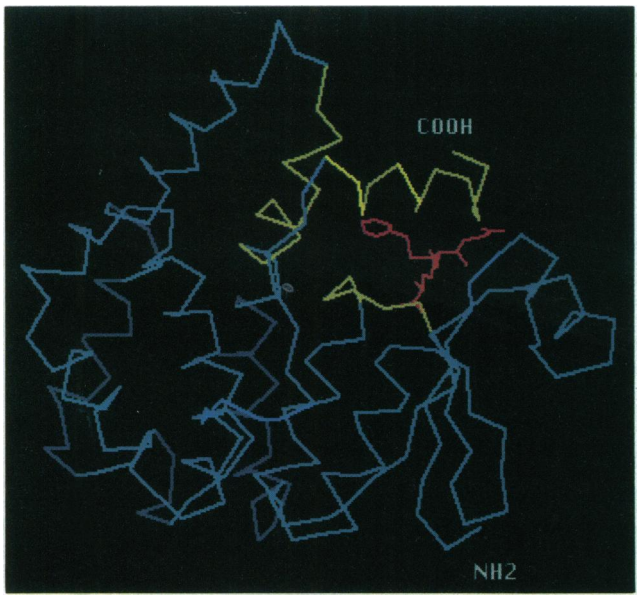

FIG. 1. Location of the electrophile binding site of an alpha class glutathione *S*-transferase: α-Carbon tracing of human glutathione *S*-transferase A1-1. The coordinates used were from Sinning et al. (18) in file 1GUH in the Brookhaven Protein Data Base. The residues that surround the benzyl portion of the inhibitor *S*-benzylglutathione (red) are shown in yellow. These residues were targeted for mutagenesis.

Mutagenesis. Mutagenesis was performed with the Alter Sites system (Promega). Two types of mutant oligonucleotides were used for random oligonucleotide-directed mutagenesis. Two oligonucleotides, Yc(9-11)random and Yc(108-110)random, contained completely random sequences at the targeted codons. The wild-type codons were replaced with NNS, where N represents an equal mixture of all four nucleotides and S represents an equal mixture of G and C. To assess the quality of the random oligonucleotides, mutagenesis was performed and over 15 mutant clones from each pool were sequenced to identify the mutations; there was no detectable nucleotide bias in either oligonucleotide pool. Several clones from each pool were also tested for glutathione *S*-transferase activity, and catalytically active enzymes were found in each pool of mutants. The second set of oligonucleotides, Yc(9-14)spike, Yc(102-112)spike, and Yc(210-220)spike, was synthesized with a small concentration of the three non-wild-type nucleotides at each position in the targeted codons (26). These oligonucleotides were synthesized by Genosys Biotechnologies (The Woodlands, TX). The oligonucleotide Yc(9-14)spike was synthesized with 90% wild-type and 10% mutant nucleotides at six target codons, while the remaining spiked oligonucleotides were synthesized with a 5% contamination at 11 target codons. These values maximized the fraction of oligonucleotides containing one or two mutations.

Mechlorethamine Cytotoxicity. Saturated cultures of *Escherichia coli* AG-1 (Stratagene) were diluted 1 to 10 in Luria broth and grown for an additional 5 hr. The cells were then diluted into M9 minimal medium containing 2 mM isopropyl β-D-thiogalactopyranoside (200 μl of cells into 4 ml of medium) and induced for 12 hr to an OD_{590} of ≈1.4. We diluted the cells and treated them with several concentrations of mechlorethamine dissolved in dimethyl sulfoxide [total volume, 1 ml; solvent concentration, 2.5% (vol/vol)]. Cells were incubated 3 hr at 37°C and then an appropriate volume of cells was plated on agar. Plates were incubated at 37°C until visible colonies appeared (≈20 hr). Colonies were counted for cytotoxicity assay or picked and amplified for plasmid preparation to determine the deduced amino acid sequence of the H-site residues.

To select for glutathione *S*-transferase enzymes that conferred increased resistance to mechlorethamine, a population of mutant plasmids was generated through the mutagenesis procedure using one of the random oligonucleotide pools and used to transform AG-1 cells. Cells were grown for 5 hr in Luria broth in the absence of antibiotic selection, and then for 17 hr under selection with both ampicillin and tetracycline. The cells were washed twice in M9 medium and induced for 12 hr. The bacteria were then treated for 3 hr with 20 μM mechlorethamine. After treatment, cells were removed from mechlorethamine-containing medium by centrifugation, washed once, and resuspended in medium for a second 12-hr protein induction. This cycle of enzyme induction and mechlorethamine treatment was continued for six rounds, using increasing concentrations of the alkylating agent (20, 40, 150, 250, 350, and 500 μM). After the 500 μM treatment, cells were incubated for 5 hr in Luria broth, and plasmids from the surviving clonal cell growths were retrieved and used to transform wild-type AG-1 bacteria to decrease the likelihood that host-mediated resistance factors affected the selection. These cells were subjected to two more rounds of induction and treatment with 500 μM mechlorethamine.

Protein Analysis. Western blotting was performed as described (27), using the anti-rat Ya/Yc (1-1/2-2) polyclonal antiserum generously provided by Cecil Pickett (Schering–Plough Research Institute, Kenilworth, NJ). For kinetic analyses, glutathione *S*-transferase 2-2 was purified essentially as described (28).

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                1                                                                  72
Rat GST 2-2     PGKPVLHYF DGRGRMEPIR WLLAAAGVEF EEQFLKT... ....RDDLAR LRNDGSLMFQ QVPMVEIDGM KLVQTRAILN
Hum GST A1-1    AEKPKLHYF NARGRMESTR WLLAAAGVEF EEKFIKS... ....AEDLDK LRNDGYLMFQ QVPMVEIDGM KLVQTRAILN
Rat GST 3-3       PMILGYW NVRGLTHPIR LLLEYTDSSY EEKRYAMGDA PDYDRSQWLN EKFKLGLDFP NLPYLIDGSR KITQSNAIMR
Hum GST P1-1      PPYTVVYF PVRGRCAALR MLLADQGQSW KEEVVTV... .....ETWQE GSLKASCLYG QLPKFQDGDL TLYQSNTILR

               73                                                                 150
Rat GST 2-2     YIATKYNLYG KDMKERALID MYAEGVADLD EIVLHYPYIP PGEKEASLAK IKDKARNRYF PAFEKVLKSH G..QDYLVGN
Hum GST A1-1    YIASKYNLYG KDIKERALID MYIEGIADLG EMILLLPVCP PEEKDAKLAL IKEKIKNRYF PAFEKVLKSH G..QDYLVGN
Rat GST 3-3     YLARKHHLCG ETEEERIRAD IVENQVMDNR MQLIMLCYNP D..FEKQKPE FLKTIPE.KM KLYSEFLG.. K..RPWFAGD
Hum GST P1-1    HLGRTLGLYG KDQQEAALVD MVNDGVEDLR CKYISLIYTN ...YEAGKDD YVKALPG.QL KPFETLLSQN QGGKTFIVGD

               151                                                          220
Rat GST 2-2     RLSRADVYLV QVLYHVEELD PSALANFPLL KALRTRVSNL PTVKKFLQPG SQRKPLEDEK CVESAVKIFS *
Hum GST A1-1    KLSRADIHLV ELLYYVEELD SSLISSFPLL KALKTRISNL PTVKKFLQPG SPRKPPMDEK SLEEARKIFR F*
Rat GST 3-3     KVTYVDFLAY DILDQYHIFE PKCLDAFPNL KDFLARFEGL KKISAYMNCS RYLSTPIFSK LAQWSNK*
Hum GST P1-1    QISFADYNLL DLLLIHEVLA PGCLDAFPLL SAYVGRLSAR PKLKAFLASP EYVNLPINGN GKQ*
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FIG. 2. Rat glutathione *S*-transferase 2-2 sequence aligned with the sequences of three glutathione *S*-transferase enzymes for which crystal structures are known. The alignment of the three crystallized enzymes (18) considers structural features in determining the most appropriate location of breaks and insertions. The residues in boldface type are involved in the formation of the electrophile-binding site of the enzyme (18, 21–23).

Lysates were centrifuged at 25,000 × *g* for 20 min, then adsorbed to *S*-hexylglutathione-agarose. Bound enzymes were eluted with 5 mM *S*-hexylglutathione. Assays with 1-chloro-2,4-dinitrobenzene (CDNB) and cumene hydroperoxide were performed with standard conditions (29, 30).

To determine apparent kinetic constants with mechlorethamine, 1 mM [^{35}S]glutathione (13 mCi/mmol; 1 mCi = 37 MBq) was allowed to react with four concentrations of mechlorethamine ranging from 50 to 400 μM for 2 min at 37°C (2.5 min for the 50 μM mechlorethamine reactions). The 30-μl reaction mixture contained 0.1 M sodium phosphate (pH 7.4) and pure glutathione *S*-transferase at 0.2 mg/ml (wild type) or 0.1 mg/ml (mutant). The reactions were stopped with an equal volume of ice-cold 5.4 M $HClO_4$ and spun through a Micro-Spin microcentrifuge filter (0.2-μm pore diameter, nylon; Alltech Associates). The reaction products were resolved by reverse-phase HPLC [Shandon Hypersil C_{18}; 4.6 × 150 mm; 5 μm particle diameter (Alltech Associates)] using a mobile phase of 3% (vol/vol) methanol, 0.29% trifluoroacetic acid in water at 1 ml/min. Radioactive peaks were monitored with a Radiometric Flo-one detector (Hewlett–Packard). Four peaks were identified and labeled A–D in the order of elution. Peak A (retention time = 4.0 min) was shown to be glutathione on the basis of its comigration with pure glutathione, and the identification of a molecular ion $(M+H)^+$ of 308 atomic mass units in mass spectrometric analysis of material from peak A fractions (31). Peak C (retention time = 9.5 min) was identified as monoglutathionyl mechlorethamine on the basis of a molecular ion of 427 atomic mass units with a signal consistent with one chlorine atom (31). Enough material from peaks B and D (retention times 6.4 and 15.3 min, respectively) could not be collected for analysis by mass spectrometry; these were presumed to be secondary products of the reaction of water or a second glutathione molecule with the monoglutathionyl adduct in peak C. A time course demonstrated that all ^{35}S originated in peak A, which decreased in size through the course of the reaction. The next peak to appear was peak C, which later disappeared coincident with the appearance of peaks B and D. This supports our molecular assignments of the four peaks. All reactions were performed in duplicate at all concentrations and the rate of the spontaneous reaction was subtracted from the rate observed. The eight initial velocities were plotted in a Lineweaver–Burk plot.

RESULTS

Wild-Type Glutathione *S*-Transferase 2-2 Confers Resistance to Mechlorethamine When Expressed in Bacteria. Mechlorethamine is a DNA-alkylating agent primarily used to treat Hodgkin and other lymphomas, and it undergoes spontaneous rearrangement to form an aziridinium ion that is capable of covalently binding to nucleophiles (32). A mixed population of equal numbers of induced cells containing either the glutathione *S*-transferase expression plasmid (pAMG207) or the control plasmid (pAMG88) was treated with solvent or mechlorethamine and then plated. We isolated plasmid DNA from the surviving cells and determined the number of glutathione *S*-transferase-positive and -negative colonies. At the two highest concentrations of mechlorethamine tested, a significant enrichment in colonies expressing glutathione *S*-transferase was obtained (Table 1). This indicated that wild-type glutathione *S*-transferase 2-2 conferred resistance to mechlorethamine in bacteria and suggested that it would be possible to select for any mutants that conferred additional resistance.

Identification of Mutant Glutathione *S*-Transferases That Confer Increased Resistance to Mechlorethamine. Mutant oligonucleotides were used as primers in mutagenesis reactions to generate a large library of mutant cDNAs in plasmid pAMG207. Bacteria harboring these plasmids were subjected to eight rounds of enzyme induction and treatment with increasing concentrations of mechlorethamine, ranging from 20 to 500 μM. After the final treatment, cells were plated on agar plates and plasmids from individual colonies were sequenced to determine the deduced amino acid sequence of the mutated regions (Table 2).

Table 1. Treatment of a heterogeneous population of bacterial cells with mechlorethamine

Mechlorethamine conc., μM	No. of colonies GST$^-$	No. of colonies GST$^+$	*P*
0	11	14	—
5	7	20	<0.2
50	4	24	<0.02
500	3	24	<0.01

Cultures of cells that either expressed glutathione *S*-transferase or did not were grown and induced using the cytotoxicity conditions. Mixtures of equal numbers of cells were treated with solvent or mechlorethamine and plated on agar. Individual colonies were picked and scored for the presence of the glutathione *S*-transferase-positive (GST$^+$) or -negative (GST$^-$) plasmid. *P* is the probability that there are equal numbers of the two types of colonies after mechlorethamine treatment.

Clones of cells containing the different selected plasmids were induced for 12 hr and then treated with various concentrations of mechlorethamine to determine the relative resistance conferred by each mutant glutathione *S*-transferase. The mutants conferred resistance at levels ranging from 5.9- to 31.1-fold relative to cells without recombinant glutathione *S*-transferase, and the highest resistance mutant, 9-11s1, conferred 9.4-fold more resistance against this alkylating agent than did the wild-type glutathione *S*-transferase 2-2 (Fig. 3, Table 2).

Analysis of Wild-Type and Mutant Enzymes. To determine why mutant glutathione *S*-transferases could confer greater resistance, we measured kinetic constants for the conjugation of mechlorethamine by both the wild-type and mutant enzymes. To study the reaction kinetics of the labile mechlorethamine molecule, we developed an HPLC assay to quantitate mechlorethamine conjugation to [^{35}S]glutathione. The determined catalytic constants demonstrate that eight of the selected mutant enzymes are 4- to 6-fold more efficient than the wild-type enzyme at catalyzing conjugation of the mechlorethamine substrate (Table 3). The increases in catalytic efficiency were the result of 3.7- to 14.6-fold increases in k_{cat} generally accompanied by nonsignificant changes in the K_m for

Table 2. Amino acid sequences of mutant enzymes and resistance conferred against mechlorethamine

Plasmid or enzyme	Codons 9–11 sequence	Relative resistance
pAMG88a	No enzyme	1
pAMG207a	Phe-Asp-Gly (wild-type)	3.3
9-11s1	Ala-Lys-Ile	31.1
9-11s4	Val-Cys-Ile	27.6
9-11s7	Met-Lys-Ile	23.8
9-11s9	Val-Arg-Ile	17.2
9-11s15	Gly-Ile-Leu	5.9
9-11s16	Val-Pro-Leu	6.9
9-11s21	Val-Ile-Cys	7.1
9-11s22	Cys-Asp-Ile	17.1
WCs3	Leu-Asp-Glu	14.9

Random mutagenesis was performed on residues at the electrophile-binding site. Bacteria expressing this library of mutant enzymes were treated with increasing concentrations of mechlorethamine. The plasmids from surviving clones were sequenced and reintroduced into bacteria to determine the relative resistance to mechlorethamine conferred by the mutant enzymes.

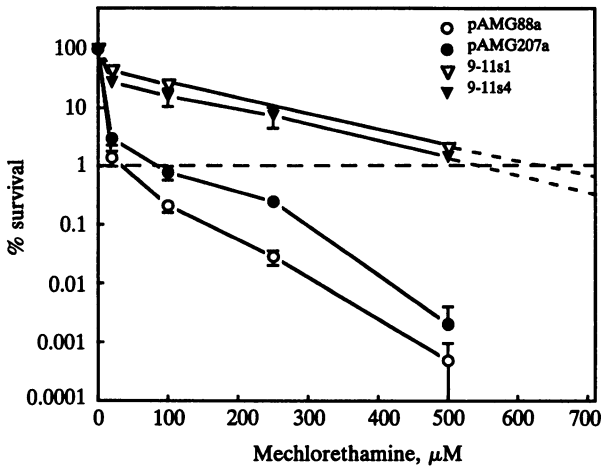

FIG. 3. Ability of mutant glutathione *S*-transferases to confer resistance to mechlorethamine. Cultures of cells synthesizing the mutant enzymes were grown for cytotoxicity assays. Cytotoxicity curves represent the percent survival compared with solvent-treated cells at various drug concentrations. Survival curves are shown for clones pAMG88a (glutathione *S*-transferase-negative), pAMG207a (glutathione *S*-transferase-positive), 9-11s1, and 9-11s4. Relative resistance values were measured from the linear portion of each curve, using a horizontal line (broken line) generally drawn at 1% (or LD_{99}) survival; lines drawn at other percent survival values would give slightly different fold resistance values. Extrapolations of the 9-11s1 and 9-11s4 curves (broken lines) were used to determine relative resistance.

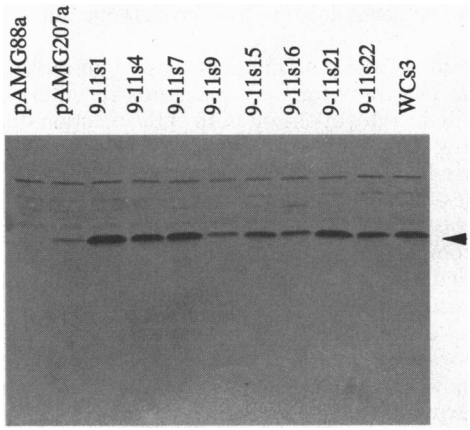

FIG. 4. Expression level of mutant glutathione *S*-transferase enzymes. Lysates of bacterial protein were made from induced cells. Western blotting was performed with an anti-rat glutathione *S*-transferase 2-2 polyclonal antiserum. Western blot analysis demonstrates the steady-state levels of expression of the wild-type and mutant glutathione *S*-transferase enzymes in bacterial culture (arrowhead).

mechlorethamine. The reaction efficiency for conjugation of the common glutathione *S*-transferase substrates, CDNB and cumene hydroperoxide, were also determined (Table 3) to see if modification of the H site to better accommodate mechlorethamine would have a discernible effect upon reaction efficiency for other substrates. The catalytic efficiency constants for these reactions demonstrated that the efficiency for conjugation of these substrates was reduced for all nine mutants. The change in substrate selectivity ratio (33) demonstrates that the wild-type enzyme's preference for CDNB over mechlorethamine is reduced by as much as 50-fold in the mutant glutathione *S*-transferases (Table 3).

When the expression level of the mutant enzymes was originally being verified, the steady-state levels of both wild-type and mutant glutathione *S*-transferases present in bacterial cells were determined. Lysates of induced bacterial cultures were made for each of the clones, and an equal amount of protein was loaded on a polyacrylamide gel. Western blot analysis (Fig. 4) showed that each of the mutant proteins was expressed to a higher steady-state level than was the wild-type enzyme. This result provides a plausible explanation for the ability of the mutant 9-11s15, which is not catalytically improved, to confer more resistance than the wild-type enzyme.

DISCUSSION

Glutathione *S*-transferases in mammalian cells catalyze the conjugation, and thus, the detoxication of a structurally diverse group of electrophilic environmental carcinogens and alkylating drugs, including the antineoplastic nitrogen mustards. We proposed that structural alteration of the nonspecific electrophile-binding site would produce mutant enzymes with increased efficiency for detoxication of a single drug and that these mutants might serve as useful somatic transgenes to protect healthy human cells against single alkylating agents. Random mutagenesis of the glutathione *S*-transferase substrate-binding domain, followed by selection of bacteria expressing the enzyme library with the nitrogen mustard mechlorethamine, yielded mutant enzymes that show significant improvement in catalytic efficiency for mechlorethamine conjugation and that confer greater resistance than the wild-type enzyme. The results suggest a general strategy for modification of drug and carcinogen-metabolizing enzymes to

Table 3. Apparent kinetic constants of wild-type and mutant enzymes determined with mechlorethamine

	Mechlorethamine			CDNB	CumOOH	
Enzyme	k_{cat}, s^{-1}	K_m, mM	k_{cat}/K_m, $s^{-1}\cdot M^{-1}$	k_{cat}/K_m, $s^{-1}\cdot M^{-1}$	k_{cat}/K_m, $s^{-1}\cdot M^{-1}$	Selectivity ratio*
Wild type	0.019 ± 0.004	0.144 ± 0.060	129 ± 45	14,300 ± 800	6870 ± 1270	110
9-11s1	0.278 ± 0.080	0.406 ± 0.143	684 ± 139	8,440 ± 420	2690 ± 710	12.3
9-11s4	0.108 ± 0.010	0.167 ± 0.026	647 ± 81	5,490 ± 420	4260 ± 640	8.5
9-11s7	0.204 ± 0.028	0.333 ± 0.060	612 ± 71	5,910 ± 420	4080 ± 820	9.7
9-11s9	0.139 ± 0.016	0.237 ± 0.041	586 ± 75	4,850 ± 1270	2580 ± 700	8.3
9-11s15	—†	—†	78 ± 12	1,030 ± 40	600 ± 240	13.2
9-11s16	0.071 ± 0.010	0.090 ± 0.032	789 ± 253	4,010 ± 170	2160 ± 500	5.1
9-11s21	0.095 ± 0.019	0.174 ± 0.059	545 ± 147	1,860 ± 80	1890 ± 350	3.4
9-11s22	0.105 ± 0.015	0.153 ± 0.038	686 ± 142	3,540 ± 210	2690 ± 720	5.2
WCs3	0.081 ± 0.015	0.128 ± 0.045	635 ± 189	1,430 ± 170	570 ± 420	2.3

Kinetic constants for wild-type and mutant enzymes with CDNB and cumene hydroperoxide (CumOOH) were determined by standard procedures (27). Catalytic activity for mechlorethamine was determined by reaction of glutathione with mechlorethamine followed by chromatographic separation of the conjugates (31).

*The substrate selectivity ratio is the ratio of k_{cat}/K_m for CDNB to k_{cat}/K_m for mechlorethamine.

†The k_{cat} and K_m values could not be determined for the mutant 9-11s15 because of the low specific activity.

achieve desired resistance in both prokaryotic and eukaryotic cells.

All but one of the nine mutants showed an increase in the k_{cat}, or turnover rate, for the conjugation reaction (Table 3). Increases in the rates of one or more of the reaction steps which follow drug binding, such as bond formation or product release, are logical targets for improvement through a forced evolution strategy, particularly in light of the results from Bolton *et al.* (34), which showed an extremely stable enzyme–product complex, with a $t_{1/2}$ of 3.5 hr at pH 6.5 for release of the melphalan-glutathione conjugate from the glutathione *S*-transferase active site.

Because several of the mutants that we identified contained a leucine or isoleucine at position 11, we constructed a single mutant in which glycine-11 was changed to an isoleucine. Bacteria expressing this mutant enzyme exhibited a sensitivity to mechlorethamine similar to that of the wild-type enzyme. The specific activity of this G11I mutant towards mechlorethamine was also similar to that of the wild-type enzyme (31). This result demonstrates that a complex interplay must exist between the three residues (residues 9–11) that are altered in the mutant enzymes that we have identified. A second unresolved point is why each of the nine enzymes containing a mutation in residues 9–11 was found at significantly higher levels than the wild-type enzyme in the bacterial host cells. It is clear that the elevated levels of expression are also important in the increased resistance observed with the mutant glutathione *S*-transferases that we have identified. In fact, the data with the 9-11s15 mutant demonstrate that elevated expression alone is able to confer increased resistance—however, not to the same extent as the mutants that are both expressed to a greater level and catalytically improved. We are unable to perform the converse experiment with a catalytically improved enzyme that is expressed to levels identical to the wild-type enzyme, as no appropriate mutants have been identified. Such an experiment would allow us to segregate the effects of elevated expression and elevated catalytic activity.

The effectiveness of cancer chemotherapy is generally limited by toxicity to the patient. Several authors have suggested that one way to allow the use of increased drug doses would be to express recombinant detoxication enzymes in human bone marrow cells by using somatic gene therapy (2, 10, 35–38). The strategy described in this report enables the production of detoxication enzymes that are designed specifically to confer resistance to individual antineoplastic agents. We have shown here that it is possible to generate mutant glutathione *S*-transferase enzymes that confer substantially increased resistance to mechlorethamine. The existent library should also be amenable to screening with other alkylators used in cancer chemotherapy, for which hematopoietic stem cell toxicity is known to be dose-limiting. As a general strategy for the design of somatic transgenes, generating enzymes that are 5 to 10 times more efficient may prove to be a more efficacious way to protect healthy cells than by seeking higher levels of the wild-type gene product. This approach would complement current approaches in somatic gene therapy that include increasing transgene copies per cell and the use of strong gene promoters to express the transgene. Selection for more efficient enzymes is an approach that could also be used to generate mutant forms of other detoxication enzymes, such that an arsenal of defense enzymes could be created to protect diverse cell types of clinical or commercial importance against a variety of specific electrophiles.

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